Aldose reductase and the role of the polyol pathway in diabetic nephropathy

MARJORIE DUNLOP

Department of Medicine, Royal Melbourne Hospital, University of Melbourne, Parkville, Victoria, Australia, 3050

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Background. In diabetic renal complications, hyperglycemia may cause damage at a cellular level in both glomerular and tubular locations, often preceding overt dysfunction. Our previous work has implicated aldose reductase in a pathway whereby aldose reductase-induced use of nicotinamide adenine dinucleotide phosphate (reduced form) (NADPH) drives the pentose phosphate pathway, which culminates in a protein kinase C–induced increase in glomerular prostaglandin production and loss of mesangial cell contractility as a possible cause of hyperfiltration and glomerular dysfunction in diabetes. In this model, aldose reductase inhibition in vitro redresses all aspects of the pathway proposed to lead to hyperfiltration; aldose reductase inhibition in vivo gives only a partial amelioration over the short-term or is without effect in the longer term on microalbuminuria, which follows glomerular and tubular dysfunction. In diabetes, hyperglycemia-induced renal polyol pathway activity does not occur in isolation but instead in tandem with oxidative changes and the production of reactive dicarbonyls and α,β-unsaturated aldehydes. Aldose reductase may detoxify these compounds. We investigated this aspect in a transgenic rat model with human aldose reductase cDNA under the control of the cytomegalovirus promoter with tubular expression of transgene.

Methods. Tubules (S3 region-enriched) from transgenic and control animals were prepared, exposed to oxidative stress, and analyzed to determine the cellular protein dicarbonyl content.

Results. In tubules from transgenic animals, oxidative stress–induced dicarbonyls were significantly reduced, an effect not seen when an aldose reductase inhibitor was present.

Conclusions. Aldose reductase may both exacerbate and alleviate the production of metabolites that lead to hyperglycemia-induced cellular impairment, with the balance determining the extent of dysfunction.

The Diabetes Control and Complications Trial [1] and the United Kingdom Prospective Diabetes Study [2] strongly implicate hyperglycemia in the pathogenesis and progression of microvascular complications in type 1 and type 2 diabetes. The clinical course of diabetic nephropathy has been described in detail and a grading system for the disease has been elaborated [3, 4]. However, the mechanism(s) by which hyperglycemia causes these complications remains controversial. This is because of the interrelated nature of the metabolic pathways used in the presence of the raised level of glucose, most importantly, in tissues in which glucose uptake does not depend on insulin and glucose is converted to both enzymatic and nonenzymatic products [5]. The enzyme aldose reductase (AR), which catalyzes the reduction of glucose to sorbitol in the polyol pathway, has been extensively studied for a potential role in the development of microvascular complications, including early diabetic nephropathy. A published finding of an increase in aldose reductase mRNA in patients with type 1 diabetes and nephropathy but not in patients with diabetes without nephropathy, is consistent with the degree of aldose reductase gene expression that modulates the risk for nephropathy [6]. However, in human studies, aldose reductase inhibitors have only a partial effect in ameliorating renal microvascular complications. A study of 6 months’ administration of an aldose reductase inhibitor had an effect on hyperfiltration in the presence of normal albuminuria [7], other studies have altered the course of microalbuminuria [8] whereas several others have proven negative [9, 10]. Such negative findings have fueled a controversy surrounding the contribution of the aldose reductase enzyme but are difficult to interpret, considering the multiple cell types within renal tissue, potential differences in aldose reductase expression, the long time-course for development of complications, and the relatively short length of human trials and variable potency and penetration of inhibitors. Moreover, those factors that perpetuate or extend changes in damaged tissues may differ from the initial pathogenic mechanisms and the latter may not be addressed using intervention studies. The hyperglycemia-related factors that may initiate the early renal functional changes of hypertrophy, hyperfiltration, and subsequent microalbuminuria have most often focused on the glomerulus. However, both glomerular and tubular impairment may coexist. Acute tubular epithelial cell hypertrophy contributes to diabetes-modi-
fied renal growth [11]. Additionally, significantly increased excretion of proximal tubule proteins can be found in type 1 diabetes, whereas albuminuria, a marker of glomerular impairment, remains within the normal range [12]. Progressive diabetic kidney disease involves glomerular, tubular, and tubulointerstitial injury [13]. The interrelated pathways that may contribute to early pathogenesis in the presence of raised glucose levels in the kidney are the aldose reductase (polyol formation) pathway, de novo synthesis of diacylglycerol, nonenzymatic glycation, glucose autoxidation, and dicarbonyl or lipid peroxidative stress.

**Aldose reductase (polyol formation) pathway**

The polyol pathway involves two enzymatic reactions: the first is the reduction of glucose to sorbitol by the action of aldose reductase and the second oxidation of sorbitol to fructose by the action of sorbitol dehydrogenase. Several studies on the renal location of aldose reductase have been published. Despite some discrepancies, a general consensus exists that immunoreactive rat aldose reductase is greatest in the medulla at the inner stripe of the outer medulla, the inner medulla, and at the papillary tip [14]. Although relatively little immunoreactive aldose reductase is observed in the cortex or the proximal tubules, aldose reductase activity is reported in both the cortex and outer medulla [15]. In renal mesangial and proximal tubule cells, the accumulation of sorbitol can be demonstrated by elevated glucose concentrations; its accumulation has been proposed as a mechanism for altered cellular myoinositol level [16] and reduced Na⁺/K⁺-ATPase activity [17], each with a potentially detrimental effect in diabetes. However, in the cells of the inner medulla, sorbitol may function, together with betaine [18] and glycerophosphorylcholine [19], as part of the organic osmolyte defense against extracellular solute fluctuations. Fructose, the second product of polyol conversions, provides the conditions for a constant throughput of glucose to provide pentose phosphate pathway intermediates. Flux through the pentose phosphate pathway may be favored further if an increased NADH:NAD⁺ ratio inhibits the NAD⁺-requiring enzyme glyceraldehyde-3-phosphate dehydrogenase, preventing 1,3-bisphosphoglycerate formation from glyceraldehyde-3-phosphate in glycolysis [25, 26]. These pathways are summarized in Fig. 1. In part, activation of the pentose phosphate pathway supplies the increased requirements for ribose 5-phosphate and NADPH for biosynthetic reactions occurring with renal hypertrophy in experimental diabetes [27].

**De novo synthesis of diacylglycerol**

It has been shown that glucose can be metabolized directly to diacylglycerol, by a process involving conversion of triose phosphate intermediates produced when glucose-6-phosphate is used by glycolysis or the pentose phosphate pathway. Production of diacylglycerol is the
presumed mechanism for the elevated protein kinase C activity observed in several tissues obtained from diabetic animals or those that are exposed in vitro to high glucose concentrations [28, 29]. We have shown that one consequence of potential importance in the pathogenesis of diabetic complications is increased prostaglandin synthesis as a consequence of protein kinase C activity. Increased phospholipase A2, supplying the arachidonic acid precursor to prostaglandins, has been shown in glomeruli and mesangial cells from diabetic rats and its increased activation attributed to activation of protein kinase C [30]. It seems likely that overproduction of vasodilatory prostaglandins by renal glomeruli plays a role in early renal hyperperfusion and hyperfiltration. A link between the aldose reductase pathway and prostaglandin synthesis is demonstrated by the reversal by aldose reductase inhibitors of increased glomerular pentose phosphate pathway activity and the protein kinase C and phospholipase A2 activation seen with raised glucose [30] and by their inhibitory effect on vasodilatory prostaglandin production in the glomerulus in experimental diabetes [31].

Nonenzymatic glycation

Nonenzymatic glycosylation of protein begins with the covalent attachment of glucose to reactive amino groups at a rate determined by blood glucose concentrations. These early glycation products (Schiff bases and Amadori products) can serve as precursors to advanced glycation products with complex glucose-derived cross-linking altering the structure and function of cells and supporting matrix [32]. Alteration in the function of various microvascular and macrovascular cells are induced by interactions with advanced glycation product-modified matrix proteins. Albumin modified by Amadori glucose adducts activates type IV collagen gene transcription in glomerular mesangial cells [33]. Plasma levels of Amadori albumin are increased in type 1 diabetes [34] and have been shown to be significantly independently associated with nephropathy classified by persistent albuminuria [35]. Aminoguanidine treatment, which reduces glomerular basement membrane–advanced glycation product content, ameliorates mesangial expansion and albuminuria [36]. Moreover, nonenzymatic glycation of reactive amino groups in model proteins increases the rate of free radical production nearly 50-fold [37], providing a link with oxidative changes described in the next sections.

Glucose autoxidation and glycoxidation

Trace metal–catalyzed oxidation of glucose can form reactive oxygen species [38]. The oxidative modification of carbohydrates, lipids and proteins that follows can produce glycoxidation and advanced glycation end products such as pentosidine, carboxymethyllysine, carboxyethyllysine, malondialdehyde-lysine and 4-hydroxynonenal and acrolein-protein adducts [39–42] as detailed below.

Free radical, oxidative, and carbonyl stress in diabetes

Considerable evidence suggests generation of free radical species in the diabetic state, which may be brought about by a coordinated increase in production and in impaired free radical scavenging. In the pathways previously discussed, free radicals may arise from multiple pathways including autoxidation of glucose, hydrogen peroxide (H$_2$O$_2$) generated from the oxidation of enediols formed from Amadori products [41] or superoxide (O$_2^·$) formed by the mitochondrial oxidation of NADH to NAD$^+$ [43] or in the formation of prostaglandins, as the PGH synthase reaction utilizes NADH (and NADPH) with the generation of O$_2^·$ [44]. Both H$_2$O$_2$ and hydroxyl radicals (OH) may be derived from O$_2^·$. Several studies show the capacity of glomerular and tubule cells to generate reactive oxygen species with potential to impose a free radical stress [45, 46]. This stress may be modified depending on the effective disposal of free radical species within specific cell types [47, 48]. Levels of H$_2$O$_2$ may be decreased by the action of catalase or glutathione peroxidase. This latter enzyme requires an effective cellular level of reduced glutathione. Regeneration of reduced glutathione requires and it has been contended that as aldose reductase-catalyzed reduction of substrates, including glucose, uses NADPH, this places a demand on the cellular glutathione system for reduction of free radicals. This would apply to all aldose reductase-catalyzed reactions. The concept of oxidative stress, as distinct from carbonyl stress, has received considerable interest in diabetic vascular complications and investigations have been concerned primarily with the formation of intracellular adducts of lipid and glucose products, their mode of production, and the contribution to advanced glycation products and cellular stress. This has been the subject of two recent extensive reviews [39, 40]. The distinction between oxidative stress and carbonyl stress stems from whether cellular damage is imposed by oxidative products or occurs nonoxidatively [39]. Dicarbonyl species formed without oxidation include methylglyoxal, which forms nonoxidatively from triose phosphates and 3-deoxyglucosone, itself formed by the nonoxidative 1,3-enolation of Amadori adducts and from fructose. From these active precursors, glycated or glycoxidated protein adducts are formed contributing to the advanced glycation end (AGE) products of diabetes [39–42]. Carboxymethyllysine adducts of protein have been shown recently to be signal-tranducing ligands for one receptor recognizing advanced glycation end products, the receptor for advanced glycation end product (RAGE), with receptor-dependent modification of gene expression [49]. This follows the demonstration of elevated serum levels of carboxymethyllysine in serum of
patients with diabetes and the immunohistochemical localization of this marker of glycoxidation together with malondialdehyde-lysine and 4-hydroxynonenal adducts in the expanded mesangial area, capillary walls, and glomerular nodular lesions of renal biopsies from patients with type 2 diabetes and diabetic nephropathy [50] and in glomerular lesions in experimental diabetes [51].

Dicarbonyl and lipid dialdehyde substrates for aldose reductase

Of immediate relevance to any consideration of the aldose reductase pathway in hyperglycemic conditions is the findings from many sources that the dicarboxylics, methylglyoxal and 3-deoxyglucosone, which contribute to glycoxidation products, and the lipid dialdehyde products of the oxidation of unsaturated fatty acids in membrane and lipoprotein phospholipids (4-hydroxynonenal, formed by the oxidation of linoleic acid and acrolein formed by the oxidation of arachidonic acid), which contribute to lipoxidation adducts are most effective substrates for aldose reductase [52, 53] with a substrate specificity for a recombinant form of the enzyme higher than that for glucose (Fig. 2). Reactive endogenous aldehydes formed either nonoxidatively or oxidatively, including methylglyoxal, 3-deoxyglucosone, and 4-hydroxynonenal inhibit glutathione reductase [54], which are required for regeneration of reduced glutathione. Thus, aldose reductase may modify formation of reactive dicarboxylic compounds and lipid aldehydes and subsequently protein-bound adducts associated with oxidative stress and tissue damage. This role for aldose reductase has not been extensively investigated in diabetes. Possibly interrelated aspects of aldose reductase with glucose-mediated metabolic changes leading to glycoxidation or lipoxidation in diabetes are outlined in Fig. 3. Because it may be possible in some tissue sites to propose a protective rather than damaging role for aldose reductase, its regulated expression may be an important determinant of the balance between these functions.

Aldose reductase expression and transgenesis

Renal osmolarity-dependent transcriptional regulation of aldose reductase has been initially described in the renal medulla [55] and subsequently shown in several cell types including the rat kidney, renal medullary cells [56, 57], and mesangial cells [58]. In rat mesangial cells, increased aldose reductase in response to glucose required hypertonic levels (~600 mOsm/kg), seen with addition of 300 mmol/L glucose, in one reported study [58]. However, in cultured cells from nonrenal sources, increased aldose reductase mRNA is seen at lower glucose concentrations. In cultured bovine endothelial cells, a glucose concentration of 18 mmol/L is effective [59] whereas in one fetal rat aortic smooth muscle cell line, A10, increased transcription follows 80 mmol/L glucose [60] and in a second line, A7r5, glucose increased aldose reductase mRNA levels in a glucose concentration-dependent manner up to 205.5 mmol/L [61]. Feeding galactose increases the alcohol product galactitol following aldose reductase activation and produces a rapid polyl-dependent regulation of aldose reductase gene expression in both the renal cortex and medulla [62]. An initial polyol-dependent decrease in renal papillary aldose reductase mRNA supports feedback regulation by the polyl product [63] but sustained feeding with 50% galactose increases whole kidney aldose reductase mRNA [64]. Streptozotocin-induced diabetes is associated with increased renal aldose reductase gene expression [65] as is diabetes of 3 months duration in the diabetes-prone BB/wor rat [66]; an implication that diabetes-related hyperglycemia or
hypertonicity stimulates aldose reductase gene expression. Characterizations of the promoter region of the aldose reductase gene have shown the presence of 5'-flanking sequence ~11 base-pair elements responding to osmotic stimuli. This osmotic response element (ORE) has been reported for rabbit [67], human [68], mouse [69], and rat [70] aldose reductase [71] each containing a C/TGGAAAATCAC ([C] cytosine: rabbit. [T] thymidine: human, mouse, and rat) nucleotide sequence conferring an osmotic response. One report indicates that in the sequence of human aldose reductase at least three ORE sequences occur, none of which in isolation can induce transcription [68]. A recent investigation of the rat aldose reductase gene by deletion analysis shows the requirement for ORE-like sequences as major cis-acting elements but shows additionally that glucose results in a greater fold induction of expression in response to glucose in comparison to other osmolytes [72]. Further, because the augmented response to glucose was not seen with non-cell permeable L-glucose or the nonmetabolizable 3-O-methylglucose, a glucose-specific induction of aldose reductase expression, dependent on glucose metabolism, was proposed by these authors. It may be of considerable relevance that, in the same smooth muscle cell type as used in those studies, aldose reductase mRNA induction is seen in response to oxidative stimuli and found after both H2O2 and 4-hydroxynonenal [73]. In the Madin-Darby canine kidney cell line, hypertonic activation induction of aldose reductase mRNA (and ORE-driven reporter gene expression) is prevented by inhibition of p38-mitogen activated protein kinase (p38-MAPK) and of mitogen-activated extracellular regulated kinase, with the indication that these two kinases are involved in ORE-dependent control of aldose reductase gene transcription [74]. Together with the involvement protein kinase C in oxidative stress conditions of raised glucose [76] my own recent investigations and those of others that show that experimental diabetes and raised glucose [77, 78] or H2O2 [78] activate p38-MAPK in glomeruli and mesangial cells, provides two potential mechanisms for glucose to contribute to an ORE-induced activation of aldose reductase expression, which may or may not require concomitant hypoxic conditions.

Two mouse lines, transgenic for human aldose reductase, have been reported. One, carrying the hAR2 cDNA...
driven by the murine major histocompatibility class (MHC) class I molecule promoter, developed thrombo-
sis in renal vessels and deposits in Bowman's capsule similar to those of the diabetic exudative lesion after 6
weeks of a normal diet [79]. However, the presence of this transgene did not alter the course of galactose feeding–
induced urinary albumin excretion [80], although transgenic animals fed a galactose diet developed cata-
racts and occlusion of the retinochoroidal vessels [79].

A second transgenic mouse line in which human AR
cDNA was linked to the mouse aA-crystallin promoter and which demonstrated an increase in lens aldose reduct-
tase shows an accumulation of sorbitol to a high level under hyperglycemic conditions and an acceleration in diabetic cataract formation [81]. A recent finding in these human aldose reductase-targeted lens showed that, in addition to an osmotic stress, the polyol pathway is a major contributor to the generation of hyperglycemic oxidative stress in lens, demonstrated by a decrease in reduced glutathione and a concomitant rise in a lipid peroxidation product, malondialdehyde [82]. As an experimental model, we have established a line of trans-
genric rats on a PVG/c strain background that express a tubule (data not shown). Some observations of the S3 proximal tubule segments that were taken from PVG/c control or Tg-CMVhAR2 rats. The assay me-
asures protein-bound 2,4-dinitrophenylhydrazone formed by a reaction of protein dicarbonyls with 2,4-dinitro-
phenylhydrazine (DNPH) after treatment with streptomy-
cin sulfate to remove contaminating nucleic acid [78].

Proteins from S3 tubules isolated from PVG/c control animals contained significantly more DNPH-reactive di-
carbonyl groups than S3 tubule segments isolated from Tg-CMVhAR2 rats. The assay measured protein-bound dicarbonyl/mg protein, N = 6; P < 0.01), each group between 12 and 14 weeks of age. When S3 tubules were exposed to H2O2 in vitro for 18 h, a significant increase in protein-bound dicarbonyls was seen only in
PVG/c control S3 tubules. In the presence of an aldose reductase inhibitor (imirestat), however, protein-bound dicarbonyls increased significantly in both PVG/c and Tg-
CMVhAR2 S3 tubule segments (Fig. 4). Thus, it could be concluded that aldose reductase expression in the trans-
genic rat has modified accumulated protein dicarbonyls and those found following an acute free radical stress.

Conclusions and future directions

Based on the preceding review, it is possible for several hyperglycemia-linked mechanisms to be proposed that
would singly or in concert contribute to the functional deficits seen in early renal complications in diabetes.
Evidence derived from experimental diabetes indicates that aldose reductase may initiate a process favoring both glycoxidative and lipoxidative changes that may be
Damaging to renal microvascular, glomerular, and tubule cells. Aldose reductase inhibition can delay or prevent
many early changes. However, when the effect of aldose reductase inhibition on microalbuminuria, the earliest
manifestation of renal impairment at a glomerular and matrix is required for proliferation and repair rather
than entry to an apoptotic pathway with cell loss [84].

Within the nephron, S3 has the highest level of lipid peroxides [85]. has low level expression of superoxide dismutase gene expression [86], and further, is the major
site of cellular damage in ischemic/reperfusion injury [87]. The S3 region has not been investigated specifically
for aldose reductase content or activity. In situ hybridiza-
tion localization studies did not show marked hybridiza-
tion in the putative S3 region for endogenous rat aldose reductase. It is possible to postulate that the expression

of human aldose reductase (hAR2) in this Tg-
CMVhAR2 transgenic rat could alter the local level of protection against glycoxidative and lipoxidative stress
in a physiologically relevant susceptible area. Some pre-
liminary data in support of this are presented below.

Carbonyl groups introduced into S3 tubule proteins
by a free radical stress

An assay of carbonyl groups in proteins was used to quantify oxidative modification of proteins in microdis-
sected S3 proximal tubule segments that were taken from
PVG/c and Tg-CMVhAR2 rats. The assay measures protein-bound 2,4-dinitrophenylhydrazone formed by a reaction of protein dicarbonyls with 2,4-dinitro-
phenylhydrazine (DNPH) after treatment with strepto-
mycin sulfate to remove contaminating nucleic acid [78].

Proteins from S3 tubules isolated from PVG/c control animals contained significantly more DNPH-reactive di-
carbonyl groups than S3 tubule segments isolated from Tg-CMVhAR2 rats (4.0 ± 0.22 vs. 2.9 ± 0.30 nmol pro-
tein bound dicarbonyl/mg protein, N = 6; P < 0.01), each group between 12 and 14 weeks of age. When S3 tubules were exposed to H2O2 in vitro for 18 h, a significant increase in protein-bound dicarbonyls was seen only in
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reductase inhibition on microalbuminuria, the earliest
manifestation of renal impairment at a glomerular and
tubule level, is investigated in experimental diabetes,
several studies, including those from our work, show this
inhibition to be effective in reducing microalbuminuria
in the short term [31] but without effect on longer-term
administration [88]. In part, this may be due to a balance
between the detrimental consequences of the glucose
metabolism through the aldose reductase pathway and
a cytoprotective effect of aldose reductase. Inhibition of
the polyol pathway can prevent the formation of sorbitol
and fructose as well as alterations in pyridine nucleotides
way inhibition may not be expected to alter the nonenzymatic formation of dicarbonyls nor the generation of free radicals from radical counteranions formed during protein cross-linking. Lipid peroxidation by free radical species may lead to a general or site-specific renal insult. Moreover, 4-hydroxynonenal, a major lipid-peroxidation-derived aldehyde, has been shown to be cytotoxic to proximal tubule cells [89], it can also be shown to be degraded in these cells [90]. Evidence is increasing that aldose reductase-mediated metabolism is an important component of 4-hydroxynonenal detoxification in several tissues [91–94] including lipid-peroxidative vascular wall injury [95]. When taken together, the potential for the polyol pathway to exacerbate or to alleviate hyperglycemia-related injury, presents a challenge to target the most appropriate tissue site, susceptible to modifications that over the long-term will provide protection against hyperglycemia-related injury and renal complications.

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Reprint requests to Marjorie Dunlop, Ph.D., University of Melbourne, Department of Medicine, Royal Melbourne Hospital, Grattan Street, Parkville, Victoria 3050, Australia.

E-mail: m.dunlop@medicine.unimelb.edu.au

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